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The Female Bitterling as a Biologic Test Animal for Male Hormone[1].

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(Plate I; Text-figure 1).

Introduction.

A number of different methods of testing for male hormone have been proposed (1) with the capon, the guinea pig, the rat, and the castrated male bitterling (2) as test animals. None of these tests has been very satisfactory. Recently Witschi (3) has described a change in the color of the bill of the sparrow after injections of male hormone and has suggested this as an indicator. The capon test is the one most commonly employed. Injections of potent material cause a growth of comb and wattles in four or five days. The measurement is not simple nor is it standardized (4). Injections do not produce constant results and for each test six animals should be used (5). Thus a considerable amount of expense, space, care, and attention are required for a single test. Castration must be complete and the bird can not be used again after a positive reaction until the comb has regressed.

It therefore seems evident that a more convenient method of detecting and measuring the male hormone would be of considerable value. The ovipositor lengthening of the female bitterling appears to furnish a simple biological reaction for this hormone, as the present experiments will indicate. This lengthening of the ovipositor of the female bitterling (Pl. I, Fig. 1) was first elicited by Ehrhardt and Kuhn (6) and, shortly after, independently by Fleischmann and Kann (7), but they ascribed this reaction to follicular hormones. It was later suggested as of possible use in diagnosing pregnancy by Kanter, Bauer, and Klawans (8). Although these authors did not definitely state that it was a test for pregnancy, their paper intimated this use so clearly that a number of laboratories accepted this viewpoint. It was soon shown that it could not be so considered (9, 10, 11), since urines from non-pregnant females, women in the post-climacteric period, and even from men gave positive reactions. Occasionally a pregnant women's urine would be negative. That this test is produced by male hormone will be shown below (12).

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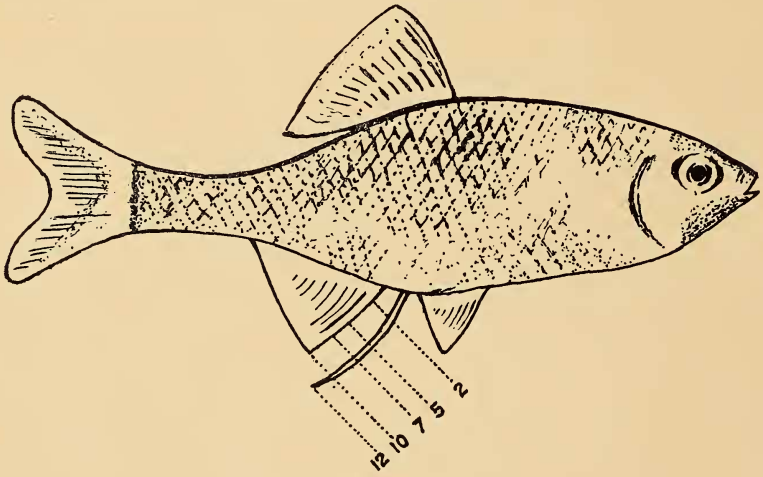
Experimental.

The female bitterling[2] develops an ovipositor which depends from the ventral margin of the body slightly anterior to the origin of the anal fin. In the quiescent state the ovipositor is seldom visible, but in those individuals in which it is visible out of season it is very small and rarely reaches 5 mm. in length. During breeding activity the organ is prolonged until it may reach 5 cm. in length (Pl. I, Fig. 2) and at spawning it is inserted into the inhalent siphon of a mussel, usually *Unio or Anodonta,* and the ova extruded into the gill-folds. Fertilization is effected by sperm which is liberated near and drawn into the inhalent siphon of the mussel and passed over the embedded ova. Hatching occurs within the gill-folds and the fry liberate themselves, in a post-larval state, two or three weeks after oviposition.

Description of Test.

To a small aquarium is added 4 liters of water, 2 liters from a stock tank and 2 liters from the tap. Two female bitterlings are placed in the tank and kept there 24 hours before introducing the material to be tested. At the end of the 24-hour period readings are made of the size of the ovipositor. The scale used is as follows: If the ovipositor is not visible the reading is 0; if the length of the ovipositor equals the length of the first ray of the anal fin the reading is 10; an ovipositor which reaches halfway down the first ray is 5; etc. (That is, the length of the ovipositor is compared to that of the first ray of the anal fin in equal units running from 0 to 10. (Text-fig. 1).) No fish with ovipositors exceeding 3 on the scale, before the addition of any test material, are used.



Text-figure 1.

Diagrammatic representation of scale used in reading length of ovipositor of female bitterling.

Ovipositor readings are taken at 24, 48, and 72 hour intervals and maximum growths under these conditions are usually observed at the second reading.

We tentatively define a bitterling unit as the amount of material which, when added to a tank containing 4 liters of water and 2 female bitterlings,

[2] We are greatly indebted to Mr. C. M. Breder, Jr., of the New York Aquarium, for advice and suggestions in respect to the fish.

produces an increase in the length of the ovipositor of one or both fish of 7 or more on the scale within 48 hours.[3] For assay it is suggested that a series of dilutions may be set up and the lowest dilution giving a positive reading may be considered to contain at least one unit, providing a positive reaction is also given in the next higher dilution.

In this work the European bitterling (*Rhodeus amarus*) was used. Kanter et al (8) used the Japanese bitterling (*Acheilognathus intermedium*) and suggest differences between the two species. However, comparative initial experiments with pregnancy urines conducted under identical conditions at the same time with both species by Coates (unpublished) did not seem to indicate any substantial difference between them.

An important observation is that female bitterlings which have been used throughout the year seem quite refractory during the late spring and summer months. From about May 15 until August 15 they react very weakly, as a rule, to preparations which at other times are definitely effective. This has been noted by other investigators. It may be a temperature effect. Gottlieb (11) working in Quebec, got good results throughout the breeding season, i. e., May-July. Whether temperature regulation or some other means may be devised to overcome this difficulty remains to be seen. In the meantime, this seasonal variation must be taken into account.

The preliminary experiments (9) already referred to, showed that urines from non-pregnant females, pregnant females, women in post-climacteric period, and men gave positive reactions in many instances. We have since extended these observations and these indicate that most normal urines from young adult males cause reactions if a large enough quantity can be used without harm to the fish. This suggested that the responsible factor might not be the follicular hormones. Moreover, the fact that urines from women 5-6 years after menopause still gave ovipositor reactions in some cases gave further support to this hypothesis. We also conducted a large number of experiments with commercial medicinal female sex hormone preparations and obtained very irregular results. Up to this point the results did not definitely indicate that the responsible factor is hormonal in nature. It might possibly be some common urinary constituent, such as creatinine, uric acid, or indican.

It was therefore seen to be necessary to determine what type of compound present in urine caused the reaction. Urine was first dialysed with the following results:

Experiment 1: Urine was obtained from a healthy male subject whose urines, previously tested, had invariably given positive results. 20 cc. was dialysed in a cellophane membrane against 40 cc. of distilled water in the refrigerator, for 24 hours. An undialysed portion was kept at the same temperature for the same period. 16 cc. of the dialysed urine, 32 cc. of the dialysate, and 16 cc. of the untreated urine were each added to 4 liters of water containing 2 fish. Positive reactions were noted in 48 hours in the tanks to which had been added the dialysed urine, as well as the control, but not in that containing the dialysate.

Experiment 2: A similar experiment was done with pregnancy urine. Similar results were obtained but it was noted that the dialysed urine was distinctly ammoniacal. This led to the suspicion that an excess of ammonium compounds might be the causative factor. An experiment was therefore conducted in which ammonium hydroxide was added to the water containing 2 fish. Results of this test proved to be negative. It thus seemed evident that the ovipositor stimulating substance is not an ammonium salt and is not dialysable. This also rules out many common urinary constituents, such as inorganic salts, creatinine, urea, uric acid, etc.

[3] This differs slightly from our originally suggested definition (12), i.e., that the ovipositor must reach the end of the fin. The present definition allows for differences in the initial length of the ovipositor.

The next step was to determine whether it belonged to any of the classes of sex hormones. Even though we had shown its incapability of being used as a pregnancy indicator, it was necessary to rule out definitely the anterior-pituitary-like hormone. This was easily done by boiling some "positive" urine and ascertaining that the resulting urine was still positive. The anterior-pituitary-like hormone ("AP-L") is heat labile.

The following experiment showed that the responsible factor is chloroform-soluble.

Experiment 3: 500 cc. of mixed urine from 20 pregnant women was treated with 20 cc. of conc. HCl and was then extracted three times with 100 cc. portions of chloroform in a separatory funnel. An emulsion formed which was cleared by the addition of 500 cc. of ether. The chloroform-ether layer was drawn off, evaporated on a steam bath, and the brown gummy residue dissolved as far as possible in 65 cc. of water containing 0.5 cc. of 10% NaOH. This was added to 7 liters of water containing 2 female bitterlings. In 16 hours both fish showed marked positive reactions. 35 cc. of the extracted urine from which the chloroform had been removed gave no reaction under similar conditions in 72 hours. This experiment led us to the conclusion that the effective factor is soluble in chloroform and to the impression that it is similar to the ovarian follicular hormones.

It therefore seemed logical to expect that crystalline "theelin" or "theelol," or both, would produce this reaction. We were fortunately able to obtain a small amount of each from Dr. Edward A. Doisy and they were tested and showed very slight effects, if any, on the ovipositor.

As is well known, the chloroform and ether soluble hormones of urine comprise the male as well as the female sex hormones, i. e., androsterone and estrins. We consequently subjected urine to a rough separation of these substances, using the method of Funk, Harrow, and Lejwa (13) with the modifications of Butenandt and Tscherning (14) and Kochakian and Murlin (15). A typical experiment is the following:

Experiment 4: 18 liters of mixed male urine was made acid to Congo Red and 360 cc. conc. HCl added. The mixture was concentrated on a steam bath to 1,800 cc. 500 cc. chloroform was added and the mixture refluxed for 12 hours on a steam bath. The aqueous fraction was discarded and the chloroform extract evaporated to dryness. The gummy residue was then dissolved in ether and shaken with 2N KOH until no further color appeared in the aqueous phase. The washings were extracted with ether and the ether solutions combined, and evaporated to dryness. The residue was refluxed with 50 cc. 3N KOH in methyl alcohol for 2 hours. After cooling, 2 liters of water was added and extracted repeatedly with ether. The aqueous fraction was saved. The extract was washed with water, dilute acid, and finally again with water. This constitutes the male hormone fraction.

The aqueous fraction was treated with HCl until acid to Congo Red and an additional 20 cc. HCl were added. The mixture was heated on a steam bath for 1 hour and extracted repeatedly with ether. The ether extract was washed with water, dilute alkali, and finally with water. This yields the female hormone fraction.

Each fraction was now tested for its ovipositor-lengthening effect on the female bitterling, as follows:

Experiment 5: Ether solutions equivalent to 64 cc. of urine were placed in a mortar and the ether permitted to evaporate. The oily residue in each case was emulsified with acacia and water and added to a tank of 2 bitterlings in the usual manner. After 18 hours positive reactions were seen to have been produced by the *male fraction* and none by the *female fraction.*

That the *male fraction* actually contained male hormone was substantiated by injection of a cotton-seed oil solution of it into a capon. A posi-

tive result was seen, whereas a similar test with the female fraction was negative. Both fractions, however, produced estrogenic effects when injected into immature female mice. This harmonizes with the previous experiences of many investigators (16) who found estrogenic effects with male hormone preparations. Recent work indicates, however, that highly purified or synthetic androsterone is non-estrogenic by the vaginal cornification test (17). Hence, the estrogenic effect of our male fraction may have been due to admixture of impurities.

The question now arose as to why pregnancy urines should be more potent and more constant in their activity than urines from males and non-pregnant females. We therefore repeated the above experiment, using mixed pregnancy urines. Here again the bitterling test was positive with the male fraction and negative with the female.

The facts thus indicate that the ovipositor-lengthening factor of urine is present in the male fraction, but proof that one of the male hormones is responsible was still lacking. This gap in the proof has recently been filled by the use of *synthetic* preparations[4]. Despite the fact that our experiments were performed during the refractory season we were successful in showing the efficacy of these products. At the same time we can also report what seems to be a more suitable solvent which is harmless to the fish and which offers a much better menstruum for these sterols than the acacia which we had formerly employed as an emulsifying agent. This is propylene glycol, suggested to us by Dr. Warren M. Cox, Jr., of the Research Laboratory of Mead Johnson & Co.

Experiment 6: 4 mg. of synthetic androsterone (Schering) and 3 mg. of synthetic testosterone (Schering) were each dissolved in 5 cc. of propylene glycol (Eastman) under the influence of slight heating. Aquaria with two bitterlings in 4 liters of water had been set up the previous day. All had extremely small ovipositors (0 to 2). One liter of water was removed from the aquarium and the propylene glycol solution of the sterol added quickly and shaken vigorously. It was then added to the aquarium from which the water had been taken. A third aquarium with the same amount of propylene glycol was observed as a control.

A positive result was noted in the bitterlings exposed to both the crystalline androsterone and testosterone. Propylene glycol alone was negative. The fish exposed to androsterone were greatly weakened by this substance but the testosterone had very little systemic effect.

In the fall when the animals were found to be in a normally reactive state both of these synthetic products were re-tested. It was found that 0.8 to 1.2 mg. of androsterone (18) produced positive reactions in 48-72 hours, whereas small amounts were never effective and larger doses yielded variable results. The larger doses seemed to have the same depressing effect as was mentioned above. Crystalline testosterone also gave uniformly positive reactions at a certain dosage (0.6-0.8 mg.) (unpublished data) whereas larger and smaller amounts were usually ineffective. Again it may be stated that a positive reaction is one in which the lengthening of the ovipositor totals 7 points or more on the scale. Many of the negative tests at other dosages showed slight effects, i. e., a lengthening of less than 7. It must also be pointed out that the results with these crystalline products appeared more slowly than when urine had been used, presumably because they are in a different physical state. From these experiments *it is evident that the ovipositor-lengthening phenomenon is due to male hormones.* There can be no suspicion of admixture of urinary impurities in these synthetic products.

Confirmatory evidence has also been obtained by testing male hormone concentrates kindly furnished by Dr. Benjamin Harrow of the College of

[4] We wish to thank Schering and Company and Dr. Erwin Schwenk for the synthetic androsterone and testosterone supplied.

the City of New York. This material, an oily solution of the male hormone fraction of male urine, has an activity of 10 capon units per cc., each cubic centimeter representing 1,000 to 1,150 cc. of urine. As little as 0.1 cc. added to an aquarium of female bitterlings gives a positive reaction in 48 hours. The minimum effective dose has not yet been reached.

Technique for Assay of Urine.

In attempting to determine the amount of male hormone in normal male urine, the following procedure was employed, using normal male medical students as subjects: For each assay, 4 aquaria were set up, containing 4 liters of water and 2 female bitterlings in the usual manner. To each tank was then added 10 cc., 25 cc., 50 cc., and 100 cc. portions of urine respectively. 24 hour samples of urine were used. The smallest amount causing a positive reaction was considered to contain one unit. The number of units in a 24-hour sample was then calculated. It was noted that in many instances the urine was toxic and even fatal in amounts from 50 to 100 cc. Various methods have been tried in the attempt to detoxicate the urines and finally it was found that dialysing is all that is necessary (19). By this means dialysed urines representing as much as 200 cc. of the original may be used without any ill effects whatever. Ordinarily the procedure is the following: Measured amounts of urine, usually 200 cc., are dialysed in membranes of cellophane ("plain transparent" not "moisture-proof") against running tap water for 18-24 hours. The volumes are then measured and amounts equivalent to 10, 25, 50, and 100 cc., respectively, of the original urine are added to 4 aquaria, each of which contains 2 female bitterlings. The ovipositors of the fish must have been read on the preceding day and also just before addition of the dialysed urine. Subsequent readings are made in 24, 48, and 72 hours, and the number of units determined in the manner suggested above. The average excretion of male hormone is about 35 bitterling units per day with a range of approximately 15 to 75 b.u. (see Table I). Further work to check and enlarge this series is in progress.

TABLE I.

Daily excretion of male hormone by normal adult males.

Number of 24-hour urine specimens	Number of cc. of urine which, when added to 4 liters of water, produce a positive reaction	Bitterling units excreted daily
5	10—15	75—120
15	25—35	24—45
10	75—100	9—22

Preliminary tests have indicated the unreliability of using casual specimens for even rough quantitative work. We have noted that successive samples obtained during the day from the same individuals have been exceedingly variable in the amounts of hormone excreted and some samples are even entirely devoid of the hormone. Apparently 24-hour samples of urine are needed in order to determine the output of this hormone with any degree of accuracy.

DISCUSSION.

Evidently neither theelin nor theelol is responsible for the reaction, nor are various cholane derivatives which have thus far been tested, such as cholesterol, ergosterol, and sodium taurocholate (20). Several of these

give slight reactions, which suggest the possibility that other cholane derivatives may be found which will react as well as the male hormone. Up to the present the test seems to be specific for male hormone.

In support of this we may cite several references to the literature. Glaser and Haempel (21) compared the effect of a follicular hormone preparation with several other preparations, including a male hormone product, "testosan forte." The technique of the experiments included placing males and females in the same aquarium. The results showed slight growth of ovipositors in those females subjected to the male as well as the female hormone, although the latter gave stronger results. The data given seem inconclusive, however. Glaser and Haempel conclude that certain secondary sex characteristics are influenced by both male and female hormones. Among these are the lengthening of the ovipositor of the female bitterling and the growth of the comb and wattles of the capon. This work was subjected to criticism by Fleischmann and Kann (22). They confirmed Glaser and Haempel's contention that "testosan forte" gave positive results with the female bitterling but they are of the opinion that this is due to an admixure of follicular hormone. In support of this they state that this male hormone preparation also gives the Allen-Doisy test on the mouse. A more concentrated male hormone preparation, "Proviron" (Schering) had the same action, i.e., positive reactions with both the fish and mouse tests, whereas crystalline "Proviron" reacted negatively with both. We suggest that this negative result may have been a result of incomplete solution or improper emulsification of the substance. The authors do not state the menstruum or method employed for this purpose.

An interesting contribution has also been made by Ehrhardt and Kuhn (23). In a long series of experiments they come to the conclusion that the ovipositor-lengthening factor and the estrus hormones are not identical, although both have a number of properties in common, i.e., solubility in organic solvents, heat stability, absorption on charcoal, etc. Some points of difference are the following: in the urine of pregnant mares are large quantities of estrus hormone, but small quantities of the ovipositor-lengthening factor. Non-pregnant women, on the other hand, excrete a urine with just the opposite characteristics. Eight hundred units of technical (i.e., impure) estrus hormone give a better ovipositor-lengthening result than several thousand units of crystalline estrus hormone. The blood serum of the pregnant woman gives a strong Allen-Doisy test but a weak bitterling test. Their preparations thus did not quantitatively show parallel results when tested with the fish and with the mouse. Although they believe the two phenomena to be due to two distinct substances they are of the opinion that one is a transformation product of the other. Ehrhardt and Kuhn's work harmonize with the contention that the male hormone is the responsible factor for this phenomenon.

We propose to assay urines and other biological fluids under various physiological conditions and from pathological cases for their male hormone content by this method. The method should also be of value in assaying male hormone products to be used therapeutically.

Summary.

The lengthening of the ovipositor of the female bitterling produced by the administration of human urine has been found to be due to the male hormone present therein. Confirmation of this is shown by positive effects caused by crystalline androsterone and testosterone. A bitterling unit is provisionally defined and a method of measuring the ovipositor is described.

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EXPLANATION OF THE PLATE.

Plate I.

Fig. 1. Female bitterling, showing elongation of ovipositor: considered a minimum positive reaction.

Fig. 2. Female bitterling, showing elongation of the ovipositor in the process of natural oviposition.